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Folate-Equipped Pegylated Archaeal Lipid Derivatives: Synthesis and Transfection Properties

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Abstract: We have previously shown that synthetic archaeal lipid analogues are useful vectors for drug/gene delivery. We report herein the synthesis and gene transfer properties of a series of novel di- and tetraether-type archaeal derivatives with a poly(ethylene glycol) (PEG) chain and further equipped with a folic acid (FA) group. The synthetic strategy and the purification by dialysis ensured complete removal of free FA. The lipids were mixed with a conventional glycine betaine-based cationic lipid and the resulting formulations were tested in transfection assays after complexation with plasmid DNA. All four novel co-lipids afforded efficient in vitro gene transfection. Moreover, the FA-equipped derivatives permitted ligand/receptor-based targeted transfection; their activity was inhibited when free FA was added to the transfection medium. These novel archaeal derivatives equipped with FA-PEG moieties may thus be of great interest for targeted in vivo transfection.

Keywords: archael lipids · cell targeting · folic acid · gene delivery

Introduction

Over the last several years, both recombinant viruses and non-viral vectors have been developed for gene therapy applications. However, neither viral vectors nor current synthetic vectors fulfil all the requirements of an ideal DNA delivery system. Although current non-viral vectors are less efficient than their viral counterparts (especially in vivo), non-viral systems are nevertheless of particular interest because they offer a series of advantages (including the absence of safety and large-scale production issues). Among the synthetic carriers, cationic lipids, which form complexes, called lipoplexes, with the DNA to be transferred, are especially promising. It is however generally recognised that efficient in vivo transfection will require the development of vectors that provide lipoplexes with favourable properties such as increased stability and cell-targeting capabilities.[1,2] Attempts to optimise the lipoplex stability 1) by the incorporation of high amounts of cholesterol (to increase the lipoplex membrane rigidity) or 2) by coating the lipoplex surface with hydrophilic poly(ethylene glycol) (PEG) (to form sterically stabilised liposomes, called Stealth liposomes, with reduced interactions with blood proteins) led to an increased residency time in blood in vivo, and therefore, have permitted some passive targeting. Unfortunately, such modifications also decrease the electrostatic interactions between negatively charged cell surface residues and the positively charged lipoplexes, which is required for their internalisation by endocytosis.[3] It is thus expected that the next generation of lipoplexes should be capable of active cell targeting through ligand–receptor interactions. Indeed, equipping the lipoplexes with an appropriate ligand should have the following two advantages: 1) provide selective entry into the cells that express the corresponding receptor and 2) as the internalisation process no longer involves electrostatic interactions, allow the use of neutral lipoplexes, thereby avoiding unwanted interactions with anionic plasma or extracellular proteins and reducing the toxicity of the lipoplexes. Among the numerous ligands available (antibodies, proteins, peptides, carbohydrates) folic acid (FA) is particularly attractive because it is well known in specific drug/gene delivery.[4–8] FA is a small molecule that is readily available and non-immunogenic. It binds to specific folate receptors (FRs), in particular to those of the α subtype (FRα, $K_d \approx 10^{-10}$ M).[6]

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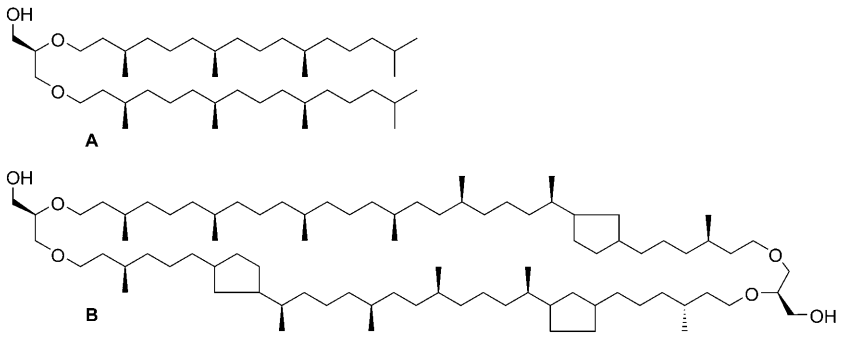
Because FRs are usually overexpressed at the surface of human cancer cells, folate-based delivery systems have been mostly used in cancer chemotherapy and medical imaging. However, as FRs are also expressed at the surface of other cell types (such as activated macrophages and epithelial respiratory cells), the therapeutic scope of cell targeting by the FA–FR interaction extends beyond cancer and includes other human diseases, for example, inflammatory diseases and cystic fibrosis.[9,10]

In previous studies we have synthesised and evaluated the properties of cationic and neutral "archaeosomes" with a view to developing gene/drug delivery systems with enhanced stability.[11,12] Archaeosomes are liposomes composed of natural lipids found in Archaea or of archaea-derived synthetic lipids that have the unique structural characteristics of archaeal membrane lipids.[13–15] Lipid components of Archaea are indeed strikingly different from those found in other forms of life and they are considered to be specific and useful markers of this evolutionary line. Apart from the archaea domain, all forms of life usually have lipids that contain ester linking groups. In contrast, archaeal lipids contain ether linkages and saturated isoprenyl units (Scheme 1).



Scheme 1. A) Natural diether and B) tetraether structures of the archaeal lipids.

The ether bonds are chemically and enzymatically more stable than esters located in comparable positions within the structures of lipids, whereas the branching methyl groups in isoprenyl units help to keep the membrane in a fluid state.

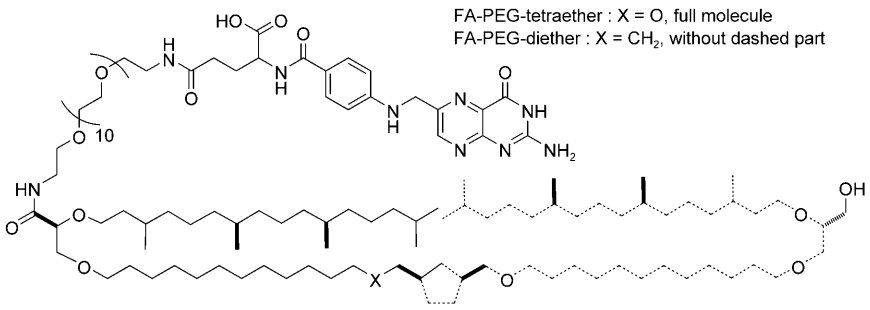
Other typical lipids from the archaea domain (Scheme 1, type B) are characterised by aliphatic phytanyl chains linked through ether units to two *sn*-2,3-glycerol units. Note that such lipids span the membrane from the inner to the outer side, thus forming monolayered membranes with increased physical stability. Archaeal membranes are therefore relatively more stable than those of other forms of life, thereby enabling these microorganisms to survive under extreme conditions of pH, temperature, pressure and salt concentration. It is thus not surprising that natural archaeal lipids have been used as innovative materials in liposome formulations with a view to exploiting their ability to increase lipid membrane stability.[16,17]

We have previously shown that synthetic archaeal lipids could significantly increase the stability of drug delivery systems even under oral administration conditions and that they are also good "helper" lipids for gene delivery applications (when compared with the commonly used dioleoylphosphatidylethanolamine (DOPE) or cholesterol).[11,12,18] We thus reasoned that it might be interesting to combine these stabilising effects with other properties desirable for in vivo transfection. Indeed, as our archaeal lipid analogues permit relatively easy chemical functionalisation,[18] they can be regarded as good anchoring points for PEG chains, equipped or not with targeting ligands at their end, to provide lipoplexes with specific targeting or stealth properties, respectively.

We thus describe herein the preparation of a series of archaeal lipid analogues with a PEG chain and their further functionalisation with a folate group. Two lipid structures (di- and tetraether) and one length of PEG chain (10 ethylene oxide units) were envisaged. The length of the PEG chain was chosen as a "not too short, not too long" compromise, the idea being 1) to facilitate the FA–FR interaction by avoiding steric hindrance problems due to the lipoplex itself and 2) to keep the preparation of lipoplexes easy despite the presence of the PEG arms. Size measurements by using dynamic light scattering were performed on both the archaeosome and lipoplex formulations. By using HeLa cells, which express high levels of FRs, we studied the in vitro transfection activity of various formulations in which the new amphiphiles synthesised herein were combined with a conventional cationic lipid derived from glycine betaine[19] and previously found to mediate significant gene transfection. In particular, the targeting ability of the formulations was evaluated by comparing the transfection activity of lipids with a folate group and lipids devoid of a targeting residue and, most importantly, by competitive inhibition assays with free FA.

Results and Discussion

Synthesis: Two types of lipids were envisaged in the present study: di- and tetraether-type structures, both of which are analogues of archaeal membrane lipids. Preparation of the diether backbone was based on the functionalisation of the readily available glycerol derivative **3** (Scheme 2).[18] Reaction of hexadecyl triflate with primary alcohol **3** gave, in the presence of proton sponge, benzyloxy diether **4** in a yield of 83%. Hydrogenolysis of the benzyloxy group provided an efficient access to alcohol **5**, which was easily converted into its acid and amine counterparts. Amine **7** was obtained in an overall yield of 45% following a three step procedure from alcohol **5**: 1) mesylation of **5** with mesyl chloride in the presence of triethylamine, 2) substitution of the corresponding



mesylate with sodium azide in DMF and 3) reduction of azide **6** by PPh_3 in THF/H_2O. Acid **8** was obtained by oxidation of primary alcohol **5** using a 2,2,6,6-tetramethylpiperidine *N*-oxide (TEMPO)-benzoate-catalysed oxidation reaction in aqueous and basic media (THF/ $NaHCO_3$ (aq)). Calcium hypochlorite was found to be the best oxidant and afforded carboxylic acid **8** in a satisfactory yield (70%; Table 1, entry 1).

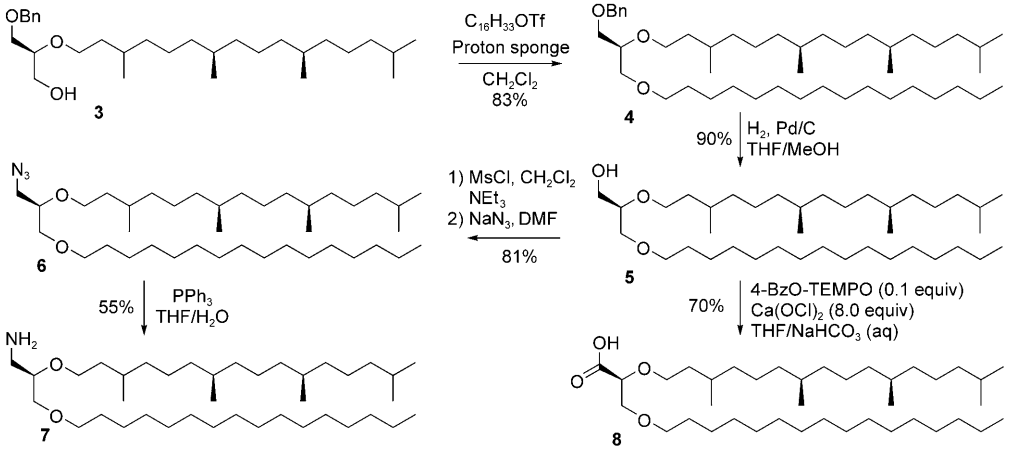
Note that a homogeneous medium had a dramatic effect on the oxidation efficiency because the use of CH_2Cl_2 instead of THF led to a lower yield of 45% after 48 h (Table 1, entry 2). The amount of aqueous phase in the reaction mixture was also found to be an important parameter as the yield dropped to 19% for a 2:1 ratio of THF/ $NaHCO_3$ (aq), even if the quantity of catalyst was increased to 0.6 equiv (Table 1, entry 3). This last result underlines the importance of favouring the hydrate intermediate for its oxidation to the carboxylic acid.

Table 1. Oxidation conditions for the preparation of acid **8**.

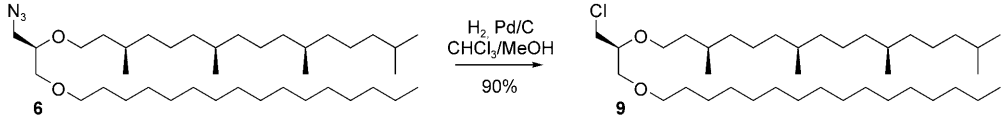
Entry	4-BzO-TEMPO [equiv]	Solvent	5% $NaHCO_3$/solvent (v/v)	Time [h]	Yield [%]
1	0.1	THF	1:1	24	70
2	0.1	CH_2Cl_2	1:1	48	45
3	0.6	THF	2:1	24	19

Interestingly, reduction of azide **6** under usual hydrogenation conditions (H_2, Pd/C), but in chlorinated solvents such as chloroform led to an unexpected product, which was unambiguously characterised as chlorinated diether **9** (Scheme 3). Note that we have already reported such a reaction with a tetraether-type azide.[18] This unexpected reaction and its mechanism are still under investigation.

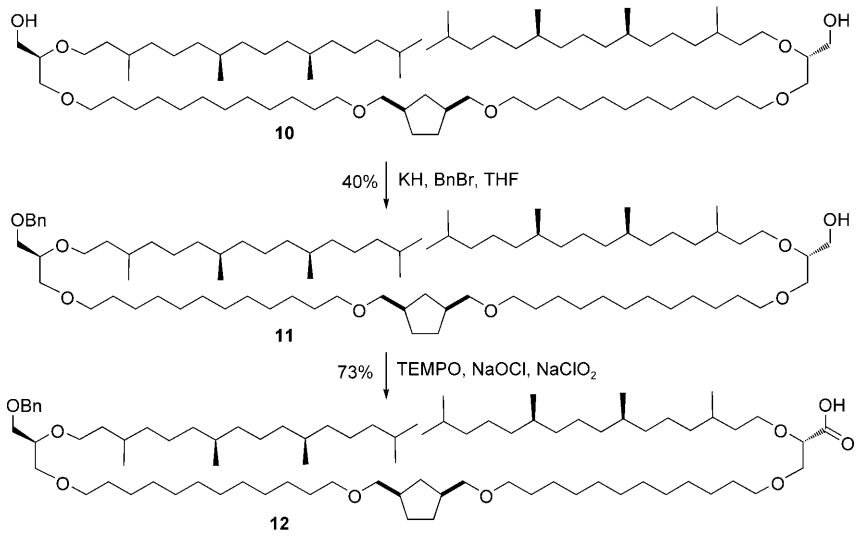
We described the tetraether backbone (diol **10**) in our previous work.[18] In this work, we envisaged its functionalisation at one end by a PEG derivative, a step that first required desymmetrisation of starting diol **10** (Scheme 4). This crucial step was carried out by an easy monobenzylation with benzyl bromide (0.9 equiv) and potassium hydride (1.0 equiv). After treatment, 40% of monobenzylated **11** was isolated in addition to recyclable starting diol **10** and the corresponding dibenzylated derivative in yields of 35 and 25%, respectively. Alcohol **11** was then oxidised in a one-pot two-step procedure under TEMPO catalysis conditions with NaOCl and $NaClO_2$ as the oxidising agents. Fine



Scheme 2. Preparation of the amine and carboxylic acid diether lipid backbones.



Scheme 3. Chlorination of the azide **6**.



Scheme 4. Preparation of the dissymmetrical tetraether backbones.

tuning of the pH during the reaction gave a clean oxidation of **11** to carboxylic acid **12** in a yield of 73%.

With acids **8** and **12** in hand, we introduced a 10-unit PEG chain into both compounds. The commercially available dissymmetrical H_2N-PEG_{570}-N_3 chain (**13**) was selected due to its ease of transformation into the primary amine by reduction. We first optimised the conditions for coupling with compound **8** and then applied the best conditions to acid **12** (Scheme 5, Table 2). The first attempts were carried out with dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) or 1-hydroxy-7-azabenzotriazole (HOAt). HOBt led to a very low yield (5%; Table 2, entry 1), whereas HOAt afforded a 50% yield of isolated **14** (Table 2, entry 2). Moving from DCC/HOAt or HOBt to a uronium salt (*O*-(benzotriazol-1-yl)1,1,3,3-tetramethyluronium tetrafluoroborate, TBTU) increased the yield to 70% for the same reaction time (24 h) (Table 2, entry 3). Stirring the reaction mixture for four days at room temperature allowed a good yield (93%) to be obtained (Table 2, entry 4).

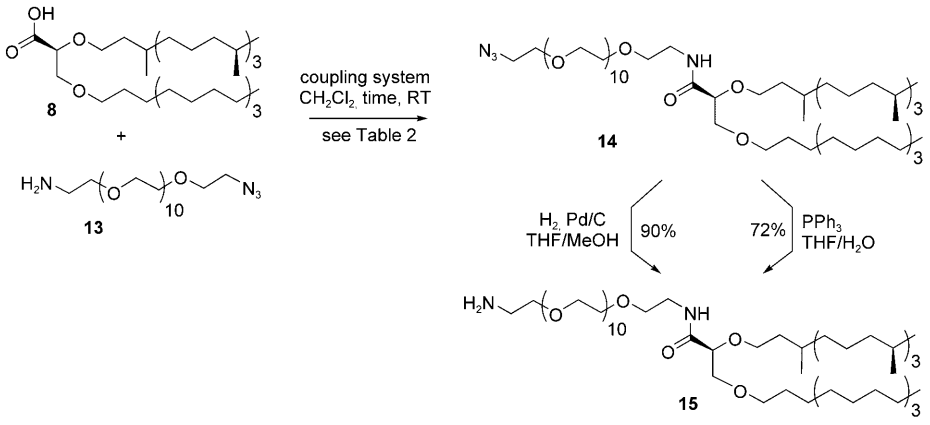
Table 2. Coupling conditions for the synthesis of the pegylated diether **14**.

Entry	Coupling system	Time	Yield [%]
1	HOBt (1.2 equiv), DCC (1.2 equiv)	24 h	5
2	HOAt (1.2 equiv), DCC (1.2 equiv)	24 h	50
3	TBTU (1.3 equiv), DIEA (1.2 equiv)	24 h	70
4	TBTU (1.3 equiv), DIEA (1.2 equiv)	4 d	93

Reduction of azide **14** was envisaged by two different procedures: 1) Pd/C-catalysed hydrogenation in THF/MeOH gave H_2N-PEG_{570}-diether **15** in 90% yield, removal of the Pd/C catalyst requiring several filtrations on a silica gel pad, and 2) reduction by PPh_3 in THF/H_2O gave **15** in a moderate yield of 72%, but with the advantage of a straightforward purification step (Scheme 5).

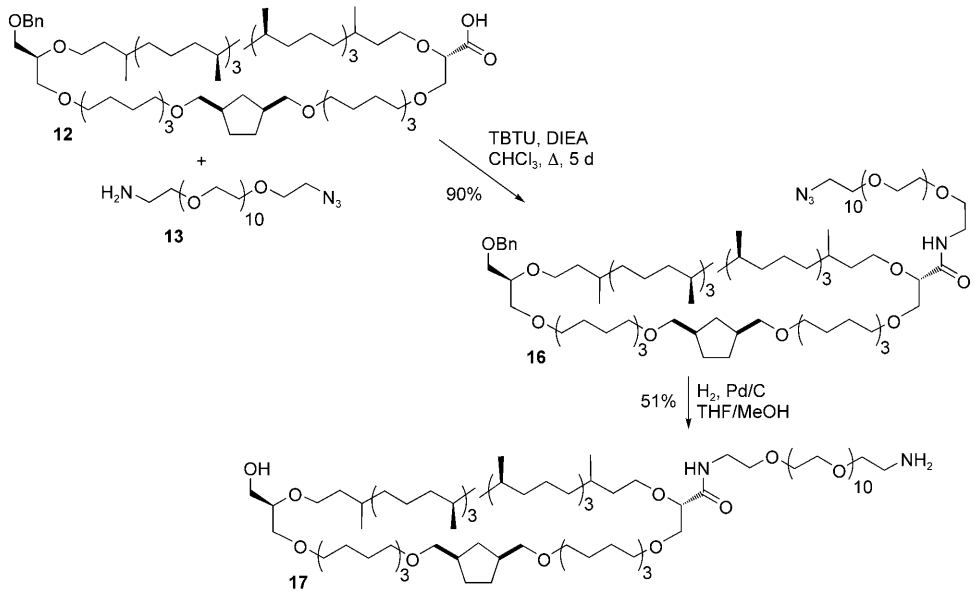
Application of the above coupling conditions to acid **12** did not provide satisfactory yields. We could however achieve almost the same efficiency when the reaction was heated at reflux in chloroform, which gave the coupling product **16** (N_3-PEG_{570}-tetraether) in a good yield (90%; Scheme 6). Hydrogenation (H_2, Pd/C) in THF/MeOH permitted both the reduction of the azide group to the corresponding primary amine and the removal of the benzyl group to give the H_2N-PEG_{570}-tetraether **17** in a yield of 51%.

The introduction of FA into the pegylated lipid amines can be expected to be associated with two main problems: 1) the poor solubility of FA and folate conjugates requires the reaction to be carried out in DMSO, which complicates the purification steps, and 2) the presence of two carboxylic acids on the glutamate moiety cannot guarantee a good regioselectivity of the coupling reaction at the α or γ position. It should however be emphasised here that coupling through the carboxylic acid groups does not affect the pteroate moiety of FA, which is crucial for the specific binding of FA to the FR.



Scheme 5. Preparation of the pegylated diether **15**.

For H_2N-PEG_{570}-diether **15**, we could promote the regioselectivity in clear favour of **19γ** (**19α**/**19γ**, 8:92)[20] because the γ position is more accessible (Scheme 7). However, a slight

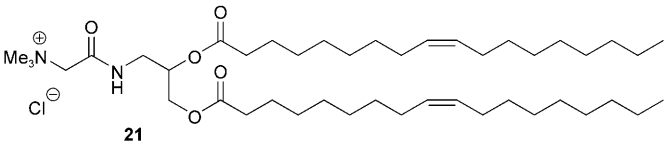


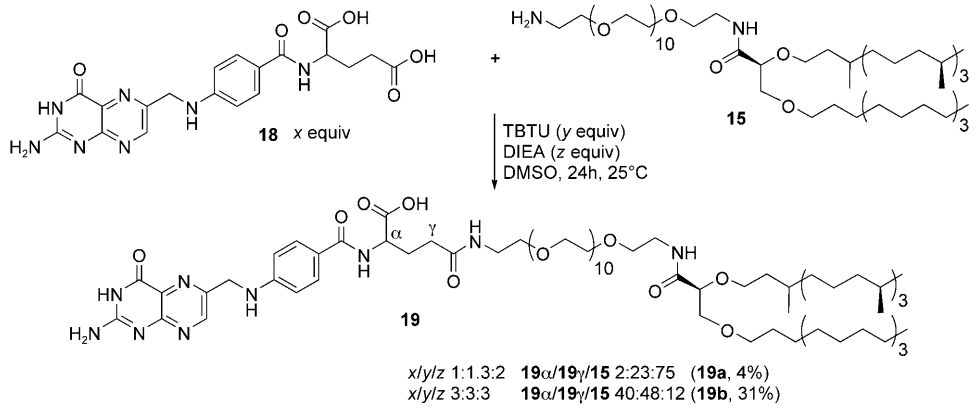
Scheme 6. Preparation of the pegylated tetraether **17**.

excess of TBTU (1.3 equiv) did not permit more than 25% conversion. Purification of FA-PEG_{570}-diether **19a** by dialysis against DMSO allowed complete removal of the free FA molecules and furnished a mixture of α-/γ-FA-PEG_{570}-diether/H_2N-PEG_{570}-diether in a 2:23:75 molar ratio (**19a**).[20] This low yield (4%) was mainly due to a loss of material during the dialysis process, the 1000 D cell-membrane cutoff was not quite adapted to the retention of all pegylated compounds. Because the positive results obtained with **19a**-based formulations in in vitro transfection experiments (see below) strongly invite us to perform, in the near future, extensive in vivo transfection studies that will require large amounts of reagent, we decided to further investigate this synthesis step to improve both the reaction yield and conversion. Thus, it should be stressed here that the use of three equivalents of TBTU/FA/DIEA (DIEA = diisopropylethylamine) significantly improved the conversion to the FA-PEG_{570}-diether. Indeed, although the dialysis purification step remained unchanged, the product was obtained in a yield of 31% and as a mixture of α-/γ-FA-PEG_{570}-diether/H_2N-PEG_{570}-diether in a 40:48:12 molar ratio (**19b**).[20] Note that these latter conditions led to a poor α/γ regioselectivity (α/γ = 45:55).

H_2N-PEG_{570}-tetraether **17** was functionalised in the presence of an excess of TBTU and DIEA (both 5.0 equiv; Scheme 8). Under these conditions and after dialysis against DMSO, H_2N-PEG_{570}-tetraether **17** was quasi-quantitatively converted into FA-PEG_{570}-tetraether **20** (α/γ = 52:48), which was obtained in a yield of 56% as a 47:44:9 mixture of the two regioisomers **20α** and **20γ** and H_2N-PEG_{570}-tetraether **17**, respectively.[20]

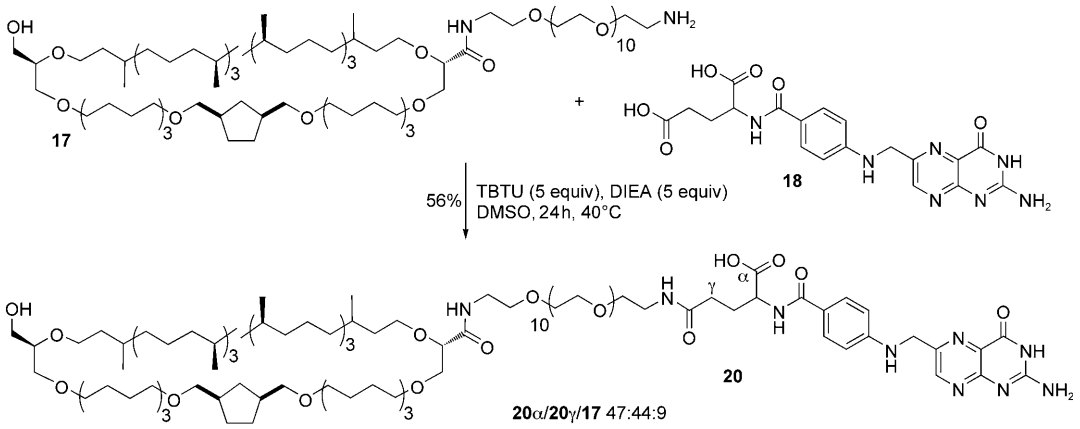
In summary, we have designed and synthesised four original pegylated di- (**15** and **19**) and tetraether-type (**17** and **20**) archaeal lipid analogues, two of which (**19** and **20**) were further equipped with a FA group at the end of the PEG chain. Note that the absence of free FA was confirmed for all samples; this is indeed critical for the evaluation of their cell-targeting capability. As already stated above, these four compounds were then used, in combination with the conventional glycine betaine-based cationic lipid **21**[19] for gene transfection experiments.





Scheme 7. Synthesis of the targeting folate derivative **19**.

Liposome/lipoplex preparation and size determination: First, liposomes and archaeosomes were prepared by hydrating lipid films composed of bilayer-forming cationic lipid **21** alone (liposomes) or combined with one bi- or monolayer-forming co-lipid **7**, **15**, **17**, **19a** or **20** (archaeosomes) with water for 12 h at 4°C followed by sonication (2 × 5 min). The sizes of the vesicles obtained were determined by dynamic light scattering. As shown in Table 3, incorporation of a



Scheme 8. Synthesis of the targeting folate derivative **20**.

Table 3. Sizes of the liposomes and archaeosomes.

Co-lipid	**21**/co-lipid (w/w)	Average diameter [nm]	Polydispersity index
none –	100:0	106	0.4
H_2N-diether **7**	95:5	373	0.8
	85:15	510	1
	70:30	827	1
H_2N-PEG_{570}-diether **15**	95:5	182	0.7
	85:15	308	0.7
	70:30	110	0.4
	50:50	214	0.2
H_2N-PEG_{570}-tetraether **17**	95:5	89	0.6
	85:15	107	0.5
	70:30	129	0.3
	50:50	139	0.3
FA-PEG_{570}-diether **19a**	98:2	111	0.4
	95:5	232	0.6
	90:10	130	0.5
FA-PEG_{570}-tetraether **20**	98:2	154	0.4
	95:5	171	0.5
	90:10	138	0.3

non-pegylated archaea-derived co-lipid, such as amine **7**, led to larger vesicles with a high polydispersity index relative to the small liposomes made solely of **21**. Note also that formulations prepared from lipid **21** and a pegylated co-lipid (**15**, **17**, **19a** or **20**) yielded vesicles of moderate size that range from 89 to 308 nm with acceptable-to-good polydispersity indexes. This may be due to a reduced aggregation of the archaeosomes coated with PEG chains. Interestingly, monolayer-forming H_2N-PEG_{570}-tetraether **17** generally yielded smaller vesicles and better polydispersities than its bilayer-forming diether counterpart **15**.

Next, lipid–DNA complexes were prepared by mixing appropriate amounts of aqueous liposome or archaeosome suspensions with plasmid DNA expressing the luciferase reporter gene (pCMV-luc, 10.6 kb). We added increasing amounts of cationic lipid **21**, lipids **21**/**7**, lipids **21**/**15**, lipids **21**/**17**, lipids **21**/**19a** or lipids **21**/**20** to a fixed amount of DNA (4 μg) to form lipoplexes with increasing (+/−) charge ratios that range from 0.5 to 8 (mean theoretical ratio of positive charges due to **21** to negative charges of the DNA phosphate groups). The lipid–DNA complexes formed were analysed after 30 min of incubation at room temperature. Size determinations led to the same conclusions as those for the corresponding liposomes. However, positively-charged lipoplexes (charge ratio >1) were generally larger (by 30–300 nm) than the corresponding liposomes (data not shown). Of note, the smallest size differences were observed with H_2N-PEG_{570}-diether **15** and H_2N-PEG_{570}-tetraether **17**, whereas the largest lipoplexes were obtained with the targeting co-lipids (**19a** and **20**).

Transfection experiments and targeting properties: The transfection activities of the various formulations were evaluated by transfecting the pCMV-luc plasmid (see above) into HeLa cells. The commercially available cationic lipid Lipofectamine was used as a positive control (at a charge ratio of 2), whereas unreacted ("naked") plasmid DNA and untreated cells were used as negative controls. Luciferase expression in the transfected cells was quantified by luminometry (MLX Dynex) and the results were expressed as relative light units (RLU) per mg of total protein (Figures 1–4).

The non-targeting di- and tetraether derivatives were first evaluated. Figure 1 shows the transfection efficiencies of cationic lipid **21** alone and combined with diether derivatives **7** and **15**. It clearly indicates that 1) the introduction of small amounts of an archaea derivative increases the transfection efficiency of cationic lipid **21** and 2) the optimal (+/−) charge ratio for these formulations lies between 2 and 4. Indeed, the introduction of more than 30% of **7** causes a decrease in the beneficial effect brought about by this co-lipid in low proportions and the presence of the PEG_{570} chain (**15**) leads to a decrease in the transfection efficiency, especially for amounts above 15%. This last observation may be due to a shielding effect by PEG. This results in a reduced accessibility of the positive charges, which may limit the electrostatic interactions required for internalisation of the lipoplexes into the cell.

The H_2N-PEG_{570}-tetraether **17** and H_2N-PEG_{570}-diether **15** formulations yielded similar transfection efficiencies

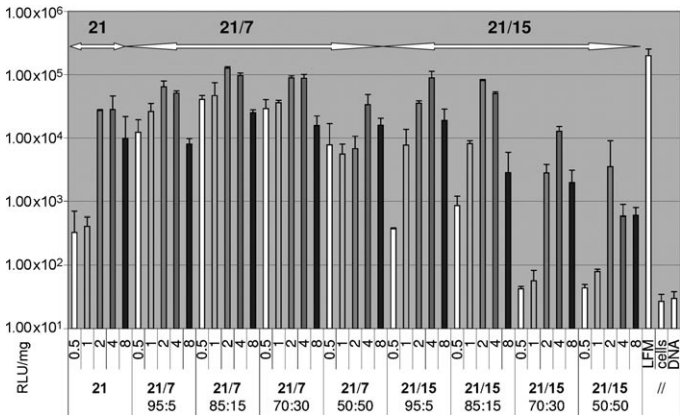


Figure 1. Transfection efficiencies for formulations of lipids **21/7** and **21/15** (H_2N-diether **7** and H_2N-PEG_{570}-diether **15**). (+/−) charge ratio from 0.5 to 8.

(both were just a bit less efficient than Lipofectamine). With regards to the beneficial effect of the addition of **17**, a limit of 15% was also observed as in the case of H_2N-PEG_{570}-diether **15** and the optimal (+/−) charge ratios were also between 2 and 4 (Figure 2). Altogether, these results confirm

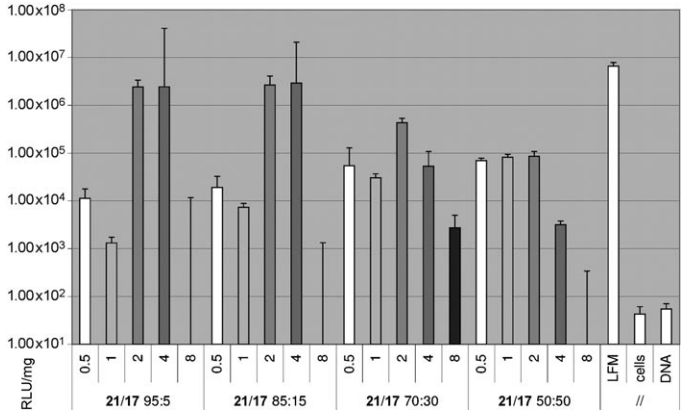


Figure 2. Transfection efficiencies for formulations of **21/17** (H_2N-PEG_{570}-tetraether). (+/−) charge ratio from 0.5 to 8.

that the addition of archaea derivatives can be beneficial for gene transfection by conventional cationic lipids. They also show that there is a limit above which the incorporation of pegylated derivatives becomes detrimental for transfection, probably because of the PEG shielding effect.

Finally, we evaluated the transfection efficiencies and targeting capabilities of the two folate co-lipids **19a** and **20** combined with standard cationic lipid **21**, the **21**/co-lipid ratio ranging from 98:2 to 90:10. Here, Hela cells were again chosen because this cell line expresses high levels of high-affinity α-folate receptors (FRα). The transfection efficiencies were determined in the same way as the non-targeting co-lipids (see above) and competitive inhibition assays were performed by adding increasing amounts of free FA (0, 1, 10, 25 and 50 nM) into the cell culture medium. In this case, only negative or slightly positive lipoplexes were tested (0.5, 1 and 2 (+/−) charge ratio) to limit as much as possible the cellular uptake of the lipoplexes by unspecific electrostatic interactions and to favour targeted folate-mediated endocytosis.[2] Indeed, as mentioned in the Introduction, efficient targeted gene transfer should have two main advantages: 1) specific targeting and 2) the possibility of using neutral lipoplexes.

Figures 3 and 4 show the transfection results obtained with the most efficient ratios of **21/19a** and **21/20**, which were 90:10 and 95:5, respectively. Interestingly, these lipid/

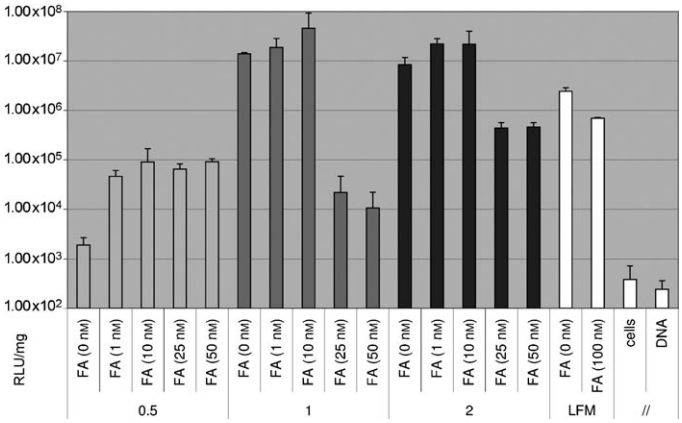


Figure 3. Transfection efficiencies and competitive inhibitions for the 90:10 formulation of **21/19a**. (+/−) charge ratio from 0.5 to 2 and concentration of FA from 0 to 50 nM.

co-lipid ratios correspond to similar molar ratios of folate-equipped lipid (di- or tetraether) in the combined system (1.26 mol% for a ratio of 90:10 of **21/19a** and 1.45 mol% for a 95:5 ratio of **21/20**). This is indicative of the proportion of ligand (FA) that is required on the surface of the lipoplexes for efficient cellular internalisation and transfection.

As shown in Figure 3, FA-PEG_{570}-diether **19a** combined with cationic lipid **21** exhibited a very high transfection efficiency for a neutral charge ratio (compared with H_2N-PEG_{570}-diether **15**, Figure 1); this activity was actually much higher than that of the Lipofectamine positive control. Most importantly, Figure 3 also shows that competitive inhibition with free FA led to highly reduced transfection on addition of FA (at concentrations equal to or higher than 25 nM) to neutral lipoplexes (charge ratio of 1). This may be due to the saturation of the FRs by free FA and suggests that the internalisation of these neutral FA-equipped lipoplexes was mainly mediated by FA–FR interactions. Moreover, note that the transfection efficiency of Lipofectamine, which is devoid of FA groups, was not affected by the addition of free FA (even at 100 nM), the internalisation process is thus being mediated here only by electrostatic interactions. In addition, Figure 3 also shows that the effect of competitive inhibition was clearly less pronounced for FA-equipped lipoplexes with a charge ratio of two (versus the neutral ones), a

fact that supports the presence of an electrostatic contribution to the transfection process under such conditions.

Although the transfection efficiencies were lower than with FA-PEG_{570}-diether formulations, the FA-PEG_{570}-tetraether formulations also displayed significant transfection activity, which was strongly inhibited by the presence of free FA (25 nM) in the cell culture medium (Figure 4 and by comparison with H_2N-PEG_{570}-tetraether **17**, Figure 2). This again supports a targeted transfection that relies on ligand/receptor-mediated internalisation. In these experiments, the transfection efficiency of Lipofectamine was again not significantly affected by the addition of free FA (100 nM) as previously found with FA-PEG_{570}-diether formulations (see above and Figure 3). Finally, the lower transfection efficiency of the FA-equipped tetraether **20** formulation (compared with **19a**) may be explained by the rigidity of the tetraether structure[21–23] (compared with the fluidity/fusogenicity of the diether derivatives[23,24]), which may increase too much the stability of the lipoplexes and thus hinder their endosomal escape, a critical transfection step known to require some fluidity of the lipoplex membranes. Such rigidity may however be quite favourable for in vivo gene transfection, in particular by the intravenous route (by increasing the lipoplex circulation time) and by aerosol delivery (by providing resistance to the shear forces of the aerosolisation process).

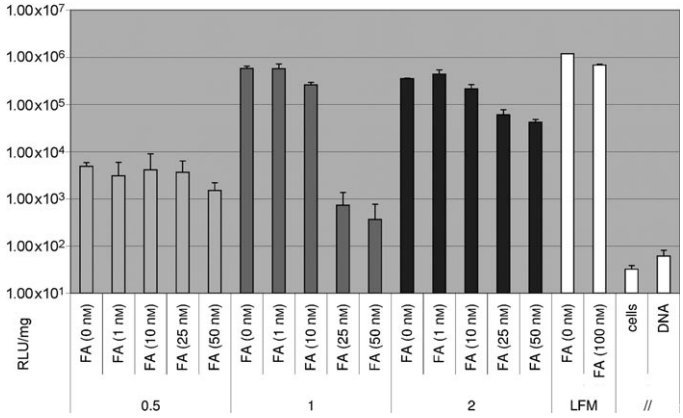


Figure 4. Transfection efficiencies and competitive inhibition for the 95:5 formulation of **21**/**20**. (+/−) charge ratio from 0.5 to 2 and concentration of FA from 0 to 50 nM.

Conclusion

We have developed a series of novel archaeal lipid derivatives that can be incorporated as co-lipids into cationic lipid formulations for gene transfection. Both di- and tetraether lipids were synthesised and functionalised with a PEG_{570} chain that was further equipped with a folate group with a view to targeted transfection. When added in relatively small proportions to a conventional glycine betaine-based cationic lipid, the non-targeting pegylated co-lipids led to a significant increase in the efficiency of gene transfection in vitro. Most importantly, neutral lipoplexes incorporating FA-equipped di- and tetraether derivatives permitted ligand/receptor-based targeted gene transfection because their activity was inhibited when free FA was added to the transfection medium. These novel lipids equipped with FA-PEG moieties may thus be of great interest for targeted gene transfection in vivo.

Experimental Section

General: Commercially available chemicals were used without further purification and solvents were carefully dried and distilled prior to use. Unless otherwise noted, non-aqueous reactions were carried out under a nitrogen atmosphere. Analytical TLC was performed on Merck 60 F254 silica gel non-activated plates. A solution of 5% H_2SO_4 in EtOH was used to develop the plates. Merck 60 H (5–40 μm) silica gel was used for column chromatography. ^1H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Bruker Avance III. MS spectra were recorded on a Waters Micromass Q-TOF equipped with a Z-spray ion source, IR spectra were recorded on a Thermo Nicolet 320 FTIR and optical rotation were recorded on a Perkin–Elmer 341 polarimeter.

1-*O*-Benzyl-2-*O*-[(7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl]-3-*O*-hexadecyl-*sn*-glycerol (4)

Hexadecyl triflate: Triflic anhydride (9.0 mL, 54 mmol, 2 equiv) was added dropwise to a stirred solution of 2,6-lutidine (6.3 mL, 54 mmol, 2 equiv) in dry CH_2Cl_2 (60 mL) at 0 °C under a nitrogen atmosphere. The reaction mixture was stirred at 0 °C for 10 min and hexadecan-1-ol (6.6 g, 27 mmol, 1 equiv) was added. After 1 h at room temperature, the reaction was quenched by the addition of water and the aqueous phase was extracted with CH_2Cl_2. The combined organic phases were successively washed with 5% aqueous HCl, 5% aqueous $NaHCO_3$ and brine, dried ($MgSO_4$) and concentrated under reduced pressure. Flash column chromatography on silica gel (petroleum ether (40–60 °C) (PE)/EtOAc, 9:1) yielded hexadecyl triflate as a brown solid (9.95 g, 26.6 mmol, 98%).

*Compound **4***: A solution of the hexadecyl triflate (9.2 g, 24.6 mmol, 3 equiv) in dry CH_2Cl_2 (10 mL) was added under a nitrogen atmosphere to a mixture of **3** (3.8 g, 8.2 mmol, 1 equiv) and 1,8-bis(dimethylamino)-naphthalene (proton sponge; 5.3 g, 24.6 mmol, 3 equiv) in dry CH_2Cl_2 (40 mL). After 4 d at reflux, 5% aqueous HCl and CH_2Cl_2 were added. The aqueous phase was extracted with CH_2Cl_2. The combined organic phases were washed with water, dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography on silica gel (PE/Et_2O, 95:5) gave compound **4** as a red oil (4.8 g, 83% in two steps). $R_f = 0.4$ (PE/EtOAc, 9:1); $[\alpha]_D^{20} = +3.7$ ($c = 1$ in CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 0.75$–0.83 (m, 18H), 1.00–1.52 (m, 52H), 3.55–3.62 (m, 6H), 3.63–3.70 (m, 2H), 3.74–3.79 (m, 1H), 4.48 (s, 2H), 7.19–7.27 ppm (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 14.14$, 19.63, 19.68, 19.75, 22.63, 22.73, 22.73, 24.37, 24.50, 24.81, 26.13, 27.98, 29.80, 32.77, 32.79, 29.38, 29.51, 29.65, 29.71, 31.93, 37.08, 37.16, 37.28, 37.35, 37.39, 37.46, 37.51, 39.36, 68.87, 70.25, 70.72, 71.65, 73.33, 77.90, 127.49, 127.58, 128.29, 138.98 ppm; HRMS (ESI): m/z: calcd for $C_{46}H_{86}O_3$ $[M+Na]^+$: 709.6475; found: 709.6472; calcd for $[M+K]^+$: 725.6214; found: 725.6224; elemental analysis calcd (%) for $C_{46}H_{86}O_3$: C 80.40, H 12.61; found: C 80.65, H 12.65.

1-*O*-Hydroxy-2-*O*-[(7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl]-3-*O*-hexadecyl-*sn*-glycerol (5): Palladium on activated carbon was added to a solution of **4** (1.6 g, 2.33 mmol, 1 equiv) in THF/MeOH (20 mL, 1:1). After 3 h at room temperature under a hydrogen atmosphere, the suspension was filtered through Celite and the solvent removed under reduced pressure. Flash chromatography on silica gel (PE/EtOAc, 95:5) gave compound **5** as a colourless oil (1.25 g, 90%). $R_f = 0.1$ (PE/EtOAc, 95:5); $[\alpha]_D^{20} = +7.3$ ($c = 1$ in CH_2Cl_2); ^1H NMR (400 MHz, $CDCl_3$): $\delta = 0.83$–0.89 (m, 18H), 1.07–1.57 (m, 52H), 2.17–2.20 (t, $J = 2.0$ Hz, 1H), 3.41–3.45 (m, 2H), 3.46–3.55 (m, 4H), 3.62–3.64 (m, 2H), 3.71–3.72 ppm (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 14.13$, 19.61, 19.68, 19.75, 22.63, 22.72,

22.69, 24.35, 24.47, 24.80, 24.10, 27.97, 29.81, 32.77, 32.79, 29.36, 29.47, 29.61, 29.70, 31.92, 37.05, 37.13, 37.28, 37.31, 37.34, 37.38, 37.44, 37.49, 39.36, 63.10, 68.63, 70.92, 71.85, 78.23 ppm; IR (neat): $\tilde{\nu}$ = 3446, 2924–2854, 1465, 1377, 1371, 1118, 715–666 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{39}H_{80}O_3$ $[M+Na]^+$: 619.6005; found: 619.5999; calcd for $[M+K]^+$: 635.5744; found: 635.5752; elemental analysis calcd (%) for $C_{39}H_{80}O_3$: C 78.46, H 13.51; found: C 78.52, H 13.56.

1-*O*-Azide-2-*O*-[(7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl]-3-*O*-hexadecane-*sn*-glycerol (6)

Mesylate: Mesyl chloride (245 μL, 3.15 mmol, 1.5 equiv) was added dropwise to a stirred solution of alcohol **5** (1.25 g, 2.1 mmol, 1 equiv) and triethylamine (446 μL, 3.15 mmol, 1.5 equiv) in dry CH_2Cl_2 (60 mL) at 0 °C. After a yellow colour had appeared, water was added and the aqueous phase was extracted with CH_2Cl_2. The combined organic phases were successively washed with a saturated aqueous solution of $NaHCO_3$ and brine, dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography on silica gel (PE/EtOAc, 9:1) gave the mesylate of **5** as a colourless oil (1.20 g, 85 %). R_f = 0.4 (PE/EtOAc 9:1); $[\alpha]_D^{20}$ = +6.4 (*c* = 1 in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.83–089 (m, 18H), 1.04–1.56 (m, 52H), 3.04 (s, 3H), 3.41–3.45 (m, 2H), 3.46–3.54 (m, 2H), 3.58–3.52 (m, 2H), 3.65–3.68 (m, 1H), 4.42–4.26 (dd, *J* = 3.6, 10.9 Hz, 1H), 4.36–4.40 ppm (dd, *J* = 3.6, 10.9 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.12, 19.64, 19.67, 19.74, 22.62, 22.71, 22.67, 24.34, 24.47, 24.78, 26.05, 27.97, 31.92, 32.77, 32.78, 27.97, 31.92, 32.77, 32.78, 29.34, 29.45, 29.56, 29.59, 29.68, 31.90, 37.26, 37.32, 37.38, 37.42, 37.47, 37.50, 39.33, 37.44, 69.03, 69.07, 69.71, 71.88, 76.37 ppm; IR (Nujol): $\tilde{\nu}$ = 1180, 1376 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{40}H_{82}O_5S$ $[M+Na]^+$: 697.5781; found: 697.5783; elemental analysis calcd (%) for $C_{40}H_{82}O_5S$: C 71.16, H 12.24, S 4.75; found: C 70.65, H 12.35, S 4.25.

Azide ***6***: Sodium azide (492 mg, 7.56 mmol, 1.5 equiv) and tetrabutylammonium bromide (812 mg, 2.52 mmol, 0.5 equiv) were added under a nitrogen atmosphere to a stirred solution of the mesylate of **5** (3.4 g, 5.04 mmol, 1 equiv) in dry DMF (10 mL). The reaction mixture was heated at reflux for 18 h, water was added and the aqueous phase was extracted with CH_2Cl_2. The combined organic phases were dried ($MgSO_4$) and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (PE/EtOAc, 98:2) to give the azide **6** as a colourless oil (2.97 g, 95 %). R_f = 0.8 (PE/EtOAc, 98:2); $[\alpha]_D^{20}$ = +3.2 (*c* = 1 in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.83–0.89 (m, 18H), 1.04–1.66 (m, 52H), 3.32–3.35 (m, 2H), 3.41–3.45 (m, 3H), 3.49–3.53 (m, 1H), 3.55–3.67 ppm (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.13, 19.59, 19.65, 19.69, 22.63, 22.72, 22.73, 24.33, 24.47, 24.81, 26.10, 27.97, 31.92, 32.77, 32.79, 29.37, 29.47, 29.60, 29.71, 31.93, 36.98, 37.00, 37.06, 37.09, 37.29, 37.35, 37.41, 37.46, 39.37, 52.05, 68.89, 70.09, 71.78, 77.86 ppm; IR (neat): $\tilde{\nu}$ = 2925–2854, 2099, 1463, 1377, 1374, 1278, 1120, 714–666 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{39}H_{79}O_2N_3$ $[M+Na]^+$: 644.6070; found: 644.6061; calcd for $[M+K]^+$: 660.5809; found: 660.5819; calcd for $[M-N_2+Na]^+$: 616.6008; found: 616.6005; elemental analysis calcd (%) for $C_{39}H_{79}N_3O_2$: C 75.30, H 12.80, N 6.75; found: C 75.20, H 12.94, N 6.80.

1-*O*-Amine-2-*O*-[(7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl]-3-*O*-hexadecane-*sn*-glycerol (7): PPh_3 (48.1 mg, 0.179 mmol, 1.5 equiv) was added portionwise to a stirred solution of **6** (74 mg, 0.119 mmol, 1 equiv) in THF/H_2O (6 mL, 1:1). After 4 h at room temperature the solvent was removed under reduced pressure. Flash chromatography on silica gel (CH_2Cl_2/MeOH, 96:4) yielded the amine **7** as a colourless oil (38.6 mg, 55 %). R_f = 0.04 (CH_2Cl_2/MeOH, 96:4); $[\alpha]_D^{20}$ = +5.7 (*c* = 1 in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.83–0.89 (m, 18H), 1.04–1.66 (m, 52H), 1.84 (br s, 2H), 2.77 (m, 1H), 2.87 (m, 1H), 3.38–3.45 (m, 3H), 3.47–3.55 (m, 3H), 3.60–3.70 ppm (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.10, 19.60, 19.67, 19.72, 22.60, 22.70, 22.67, 24.34, 24.46, 24.78, 26.10, 27.94, 29.81, 32.76, 32.77, 29.34, 29.47, 29.63, 29.68, 31.90, 37.12, 37.19, 37.26, 37.32, 37.36, 39.43, 37.47, 39.33, 43.31, 68.55, 71.14, 71.72, 79.45 ppm; IR (neat): $\tilde{\nu}$ = 3372, 2924–2853, 1463, 1378, 1357, 1117, 729–666 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{39}H_{81}NO_2$ $[M+Na]^+$: 618.6165; found: 618.6194; calcd for $[M+H]^+$: 596.6346; found: 596.6339; elemental analysis calcd (%) for $C_{39}H_{81}NO_2$: C 78.58, H 13.70, N 2.35; found: C 78.35, H 13.77, N 2.50.

1-*O*-Carboxyl-2-*O*-[(7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl]-3-*O*-hexadecane-*sn*-glycerol (8): A 5 % aqueous solution of $NaHCO_3$ (6 mL) and calcium hypochlorite (204.9 mg, 1.43 mmol, 8 equiv) were added portionwise to a mixture of **5** (106.9 mg, 0.18 mmol, 1 equiv) and TEMPO-benzoate (4.5 mg, 0.018 mmol, 0.1 equiv) in THF (6 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 15 min and then at room temperature for 20 h. The white precipitate thus formed was dissolved with aq. 10 % HCl and the aqueous phase was extracted with EtOAc. The combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. Column chromatography on silica gel (PE/EtOAc, 7:3) gave **8** as a colourless oil (77.0 mg, 70 %). R_f = 0.2 (PE/EtOAc, 7:3); $[\alpha]_D^{20}$ = +1.7 (*c* = 1 in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.83–0.89 (m, 18H), 1.02–1.57 (m, 52H), 3.44–3.52 (m, 2H), 3.65–3.71 (m, 3H), 3.78–3.82 (dd, *J* = 15.1, 4.2 Hz, 1H), 4.03–4.05 (m, 1H), 11.43 ppm (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.13, 19.56, 19.69, 19.74, 22.62, 22.72, 22.72, 24.38, 24.49, 24.80, 25.95, 27.97, 29.82, 32.80, 32.81, 29.33, 29.37, 29.48, 29.66, 29.72, 31.93, 36.51, 36.59, 36.63, 37.28, 37.36, 37.41, 37.46, 69.68, 69.72, 72.05, 78.53, 172.20 ppm; IR (neat): $\tilde{\nu}$ = 2925–2854, 1724, 1462, 1378, 1357, 1242, 1127, 937, 713–666 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{39}H_{78}O_4$ $[M-H]^-$: 609.5822; found: 609.5830; elemental analysis calcd (%) for $C_{39}H_{78}O_4$: C 76.66, H 12.87; found: C 76.20, H 12.94.

1-Chloro-2-*O*-[(7*R*,11*R*)-3,7,11,15-tetramethylhexadecyl]-3-*O*-hexadecane-*sn*-glycerol (9): Palladium on activated carbon was added to a solution of **6** (52.2 mg, 0.084 mmol, 1 equiv) in $CHCl_3$/MeOH (4 mL, 1:1). After 4 h at room temperature under a hydrogen atmosphere, the suspension was filtered through Celite and the solvent was removed under reduced pressure. Flash chromatography on silica gel (PE/EtOAc, 95:5) gave compound **9** as a colourless oil (43.9 mg, 90 %). R_f = 0.5 (PE/EtOAc, 98:2); $[\alpha]_D^{20}$ = +2.3 (*c* = 1 in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.83–0.89 (m, 18H), 1.07–1.57 (m, 52H), 3.43–3.46 (t, *J* = 6.65 Hz, 2H), 3.52–3.53 (m, 2H), 3.55–3.67 ppm (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.13, 19.61, 19.68, 19.75, 22.63, 22.72, 22.69, 24.34, 24.50, 24.80, 26.08, 27.98, 29.70, 32.77, 32.79, 29.36, 29.46, 29.59, 29.62, 29.66, 31.92, 36.91, 36.99, 37.28, 37.34, 37.38, 37.40, 37.45, 37.47, 37.49, 39.36, 43.97, 68.90, 69.99, 71.77, 78.32 ppm; IR (neat): $\tilde{\nu}$ = 2924–2854, 1464, 1377, 1366, 1123, 721–666 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{39}H_{79}O_2Cl$ $[M+Na]^+$: 637.5667; found: 637.5656; calcd for $[M+K]^+$: 653.5406; found: 653.5413; elemental analysis calcd (%) for $C_{39}H_{79}O_2Cl$: C 76.10, H 12.94; found: C 75.59, H 13.03.

***cis*-1,3-Bis(dodecan-1-*O*-{[(*R*)-tetramethylhexadecyl]-*sn*-glycerol}oxymethyl)-3-*O*-({[(*R*)-tetramethylhexadecyl]-*sn*-glycerol}oxybenzyl)cyclopentane (11)**: Under a nitrogen atmosphere, a solution of diol **10** (97 mg, 0.080 mmol, 1 equiv) in dry THF (4 mL) and benzyl bromide (9 μL, 0.072 mmol, 0.9 equiv) were added dropwise at 0 °C to a suspension of 30 % potassium hydride in oil (11 mg, 0.080 mmol, 1 equiv; previously washed twice with THF). After 2 h at room temperature, water was added slowly at 0 °C and the aqueous phase was extracted with EtOAc. The combined organic phases were dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography on silica gel (PE/EtOAc, 9:1) gave dibenzylated compound (26 mg, 25 %), diol **10** (26 mg, 35 %) and monobenzylated **11** (45 mg, 40 %) as colourless oils. R_f = 0.22 (PE/EtOAc, 9:1); $[\alpha]_D^{20}$ = +2.7 (*c* = 1 in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.80–0.90 (m, 31H), 1.03–1.41 (m, 81H), 1.41–1.63 (m, 8H), 1.70–1.77 (m, 3H), 1.92–1.99 (dt, *J* = 12.41 Hz, 8.0 Hz, 1H), 2.14–2.22 (m, 2H), 3.27–3.29 (d, *J* = 6.8 Hz, 4H), 3.67–3.40 (t, *J* = 6.8 Hz, 4H), 3.41–3.43 (t, *J* = 6.8 Hz, 4H), 3.45–3.55 (m, 7H), 3.52–3.53 (d, *J* = 1.2 Hz, 1H), 3.57–3.58 (d, *J* = 1.2 Hz, 1H), 3.60–3.62 (m, 4H), 3.65–3.68 (m, 1H), 3.69–3.74 (m, 1H), 4.55 (s, 2H), 7.27–7.34 ppm (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 19.61, 19.68, 19.75, 22.63, 22.73, 22.63, 22.73, 24.36, 26.10–26.18, 29.47–29.73, 28.85, 27.97, 29.80, 32.77, 32.79, 33.93, 37.05–37.48, 39.36, 39.71, 63.10, 68.62, 68.87, 70.27, 70.71, 70.92, 71.14, 71.66, 71.85, 73.34, 75.62, 77.91, 78.20, 127.48, 127.57, 128.29, 138.40 ppm; IR (neat): $\tilde{\nu}$ = 3473, 2925–2854, 1496, 1464, 1377, 1366, 1114, 828, 733–666 cm^{-1}; HRMS (ESI): *m/z*: calcd for $C_{84}H_{160}O_8$ $[M+Na]^+$: 1320.2011; found: 1320.2016; elemental analysis calcd (%) for $C_{83}H_{158}O_8$: C 77.33, H 12.40; found: C 77.46, H 12.47.

***cis*-1,3-Bis[dodecan-1-*O*-{[(*R*)-tetramethylhexadecyl]-*sn*-glycerol}carboxyl)-3-*O*-({[(*R*)-tetramethylhexadecyl]-*sn*-glycerol}oxybenzyl)cyclopentane**

(12): An aqueous solution of KBr (670 μL, 0.5 M) and TEMPO (1 mg, 0.006 mmol, 0.02 equiv) were added to a solution of **11** (400 mg, 0.31 mmol, 1 equiv) in EtOAc (5 mL). A 12% aqueous solution of NaOCl (200 μL) was added slowly at 0°C. After 30 min at room temperature, the reaction mixture was acidified with 5% aqueous HCl to pH < 3–4. Then 12% aqueous $NaClO_2$ (140 μL) was added slowly. The reaction mixture was stirred at room temperature for 2 h and the aqueous phase was extracted with EtOAc. The combined organic phases were washed with brine and dried ($MgSO_4$). Flash chromatography on silica gel (PE/EtOAc, 9:1 then 7:3) gave **12** as a colourless oil (297 mg, 73%). $R_f = 0.2$ (PE/EtOAc, 9:1); $[\alpha]_D^{20} = -1.0$ ($c = 1$ in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.81$–0.90 (m, 31H), 1.03–1.38 (m, 81H), 1.49–1.59 (m, 8H), 1.69–1.77 (m, 3H), 1.91–1.98 (dt, $J = 12.4$, 8.0 Hz, 1H), 2.16–2.20 (m, 2H), 3.28–3.30 (d, $J = 6.8$ Hz, 4H), 3.37–3.41 (t, $J = 6.8$ Hz, 2H), 3.43–3.44 (t, $J = 6.8$ Hz, 2H), 3.49–3.54 (m, 6H), 3.56–3.61 (m, 5H), 3.65–3.72 (m, 3H), 3.77–3.80 (dd, $J = 2.4$, 10.4 Hz, 1H), 4.02–4.06 (q, $J = 3.2$ Hz, 1H), 4.55 (s, 2H), 7.26–7.33 ppm (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta =$ 19.62, 19.63, 19.68, 19.75, 22.63, 22.71, 24.37, 24.47, 24.49, 24.80, 25.95, 26.14, 26.15, 26.18, 29.38, 29.42, 29.45, 29.52, 29.63, 29.67, 29.68, 29.71, 29.76, 28.85, 27.97, 29.82, 32.78, 32.80, 33.93, 37.11–37.52, 39.37, 39.73, 68.88, 69.94, 70.32, 70.52, 70.75, 71.14, 71.67, 72.05, 73.35, 75.61, 77.96, 78.61, 127.48, 127.57, 128.28, 138.43, 171.72 ppm; IR (neat): $\tilde{\nu} = 3582$, 2925–2854, 1733, 1496, 1464, 1377, 1366, 1260, 1114, 1029, 937, 733–666 cm^{-1}; elemental analysis calcd (%) for $C_{84}H_{158}O_9$: C 76.89, H 12.14; found: C 76.53, H 12.37.

N_3-PEG$_{570}$-diether 14: DIEA (149 μL, 0.9 mmol, 1.3 equiv) was added to a mixture of **6** (460 mg, 0.9 mmol, 1 equiv) and TBTU (321 mg, 1 mmol, 1.3 equiv) in dry CH_2Cl_2 (15 mL) under a nitrogen atmosphere. After 20 min at room temperature, a solution of N_3-PEG$_{570}$-NH_2 **13** (400 mg, 0.7 mmol, 1 equiv) in dry CH_2Cl_2 (3 mL) was added and the reaction mixture was stirred for 4 d. An aqueous solution of 1N HCl was added and the organic phase was washed with water. The combined organic phases were dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography on silica gel (EtOAc/MeOH, 9:1) gave **14** as a viscous yellow oil (941 mg, 93%). $R_f = 0.3$ (EtOAc/MeOH, 9:1); $[\alpha]_D^{20} = -4.9$ ($c =$ 1 in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.83$–0.87 (m, 18H), 1.04–1.78 (m, 52H), 3.37–3.40 (m, 2H), 3.41–3.50 (m, 4H), 3.53–3.57 (m, 4H), 3.58–3.69 (m, 43H), 3.75–3.78 (m, 1H), 3.88–3.90 (dd, $J = 2.51$, 5.92 Hz, 1H), 7.02–7.05 ppm (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 14.10$, 19.58, 19.65, 19.72, 22.60, 22.69, 22.66, 24.36, 24.47, 24.78, 26.03, 27.94, 29.84, 32.76, 32.78, 29.34, 29.46, 29.52, 29.63, 29.68, 31.89, 37.26, 37.36, 37.39, 37.45, 37.50, 39.32, 38.67, 50.63, 69.72, 69.83, 70.3–70.7, 71.47, 71.68, 80.48, 170.57 ppm; IR (neat): $\tilde{\nu} =$ 2025–2856, 2103, 1678, 1524, 1463, 1300, 1250, 1117, 720–666 cm^{-1}; elemental analysis calcd (%) for $C_{63}H_{126}O_{14}N_4$: C 65.02, H 10.91, N 4.81; found: C 64.79, H 10.95, N 4.79.

H_2N-PEG$_{570}$-diether 15: PPh_3 (157.37 mg, 0.6 mmol, 1.5 equiv) was added portionwise to a stirred solution of **14** (591 mg, 0.4 mmol, 1 equiv) in THF/H_2O (6 mL, 1:1). After 18 h at room temperature, the solvent was removed under reduced pressure. Flash chromatography on silica gel (CH_2Cl_2/MeOH, 95:5) gave amine **15** as a viscous yellow oil (327 mg, 72%). $R_f = 0.3$ (CH_2Cl_2/MeOH, 9:1); $[\alpha]_D^{20} = -6.6$ ($c = 1$ in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.82$–0.88 (m, 18H), 0.99–1.69 (m, 52H), 3.16–3.18 (m, 2H), 3.39–3.49 (m, 4H), 3.54–3.57 (m, 4H), 3.59–3.70 (m, 38H), 3.72–3.75 (m, 3H), 3.76–3.77 (m, 1H), 3.87–3.90 (m, 3H), 7.04–7.06 ppm (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 14.10$, 19.58, 19.65, 19.72, 22.61, 22.71, 22.66, 24.34, 24.45, 24.76, 26.00, 27.95, 29.77, 29.85, 32.79, 29.32, 29.45, 29.54, 20.61, 29.66, 31.88, 37.23, 37.34, 37.37, 37.43, 39.31, 38.70, 40.45, 66.89, 69.72–70.53, 71.45, 71.70, 80.47, 170.63 ppm; IR (neat): $\tilde{\nu} = 3426$, 2024–2855, 1674, 1524, 1463, 1257, 1114, 714–666 cm^{-1}; HRMS (ESI): m/z: calcd for $C_{63}H_{128}O_{14}N_2$ $[M+H]^+$: 1137.9444; found: 1137.9438; elemental analysis calcd (%) for $C_{63}H_{128}O_{14}N_2 \cdot 2H_2O$: C 64.47, H 11.34, N 2.39; found: C 64.56, H 11.18, N 2.35.

N_3-PEG$_{570}$-tetraether 16: DIEA (8 μL, 0.046 mmol, 1.3 equiv) was added to a mixture of **12** (55 mg, 0.042 mmol, 1.2 equiv) and TBTU (14 mg, 0.046 mmol, 1.3 equiv) in dry CH_2Cl_2 (2 mL) under a nitrogen atmosphere. After 20 min at room temperature, a solution of N_3-PEG$_{570}$-NH_2 **13** (20 mg, 0.035 mmol, 1 equiv) in dry CH_2Cl_2 (2 mL) was added and the reaction mixture was stirred for 6 d. An aqueous solution of 1N HCl was added and the organic phase was washed with water. The combined organic phases were dried ($MgSO_4$) and concentrated under reduced pressure. Flash chromatography on silica gel (CH_2Cl_2/MeOH, 9:1) gave **16** as a yellow oil (60 mg, 90%). $R_f = 0.2$ (CH_2Cl_2/MeOH, 9:1); $[\alpha]_D^{20} = -2.6$ ($c = 1$ in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.83$–0.89 (m, 31H), 1.04–1.45 (m, 81H), 1.48–1.56 (m, 8H), 1.71–1.76 (m, 3H), 1.91–1.98 (dt, $J = 12.4$, 8.0 Hz, 1H), 2.13–2.22 (m, 2H), 3.27–3.29 (d, $J = 6.8$ Hz, 4H), 3.37–3.39 (t, $J = 6.8$ Hz, 4H), 3.40–3.44 (t, $J = 6.8$ Hz, 2H), 3.44–3.57 (m, 10H), 3.57–3.60 (m, 7H), 3.61–3.69 (m, 37H), 3.75–3.78 (dd, $J = 2.4$, 10.4 Hz, 1H), 3.88–3.90 (dd, $J = 2.4$, 6.0 Hz, 1H), 4.55 (s, 2H), 7.04 (br s, 1H), 7.28–7.34 ppm (m, 5H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 19.62$, 19.68, 19.75, 22.63, 22.72, 24.49, 24.81, 26.06, 26.13, 26.18, 26.19, 29.52, 29.53, 29.56, 29.64, 29.65, 28.86, 27.97, 29.74, 32.79, 33.93, 37.29–37.49, 39.36, 38.71, 39.73, 50.71, 68.87, 69.86, 70.06, 70.30, 70.59–70.73, 71.10, 71.66, 71.73, 73.34, 75.63, 77.93, 80.53, 127.48, 127.56, 128.29, 138.43, 170.60 ppm; IR (neat): $\tilde{\nu} = 3583$, 3473, 2925–2855, 2103, 1676, 1523, 1463, 1376, 1350, 1257, 1114, 1029, 734–666 cm^{-1}.

H_2N-PEG$_{570}$-tetraether 17: Palladium on activated carbon was added to a solution of **16** (145 mg, 0.078 mmol, 1 equiv) in $CHCl_3$/MeOH (12 mL, 1:1). After 18 h at room temperature under a hydrogen atmosphere, the suspension was filtered through Celite and the solvent was removed under reduced pressure. Flash chromatography on silica gel (CH_2Cl_2/MeOH, 95:5) gave **17** as a colourless oil (70 mg, 51%). $R_f = 0.1$ (CH_2Cl_2/MeOH, 95:5); $[\alpha]_D^{20} = +4.4$ ($c = 1$ in CH_2Cl_2); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta = 0.83$–0.88 (m, 31H), 1.02–1.31 (m, 81H), 1.42–1.58 (m, 8H), 1.69–1.76 (m, 3H), 1.93–1.98 (dt, $J = 12.4$, 8.0 Hz, 1H), 2.14–2.22 (m, 2H), 3.27–3.29 (d, $J = 6.8$ Hz, 4H), 3.37–3.40 (t, $J = 6.8$ Hz, 2H), 3.42–3.44 (t, $J = 6.8$ Hz, 2H), 3.45–3.57 (m, 12H), 3.57–3.60 (m, 7H), 3.63–3.69 (m, 37H), 3.75–3.78 (m, 1H), 3.89–3.90 (m, 1H), 3.93–3.94 (m, 2H), 7.04 ppm (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 19.61$, 19.68, 19.75, 22.62, 22.71, 24.35, 24.46, 24.79, 26.06, 26.10, 26.18, 26.19, 29.47–29.73, 28.85, 27.96, 29.82, 29.88, 32.78, 32.81, 33.93, 37.05–37.48, 39.36, 38.72, 39.72, 63.09, 66.94, 68.62, 69.85, 70.09, 70.34, 69.70, 69.77, 69.88, 70.00, 70.17, 70.21, 70.36–70.61, 71.14, 71.72, 70.92, 71.54, 71.85, 75.62, 78.24, 80.51 ppm; IR (neat): $\tilde{\nu} = 3420$, 2923–2854, 1661, 1530, 1463, 1377, 1350, 1250, 1110, 734–666 cm^{-1}; HRMS (ESI): m/z: calcd for $C_{101}H_{202}O_{19}N_2$ $[M+H]^+$: 1748.4980; found: 1748.4943.

FA-PEG$_{570}$-diether 19: A solution of FA (**18**; 13.7 mg, 0.032 mmol, 1.2 equiv) in dry DMSO (5 mL) was heated until all material was dissolved. TBTU (16.7 mg, 0.052 mmol, 2 equiv), DIEA (10.7 μL, 0.065 mmol, 2.5 equiv) and a solution of **15** (29.6 mg, 0.026, 1 equiv) in dry DMSO (2 mL) were added at room temperature under a nitrogen atmosphere. After 48 h at 30°C in the dark, the reaction mixture was lyophilised. The residue was purified by dialysis (molecular weight cut-off (MWCO) = 1000 D) against DMSO to afford **19** as a yellow solid mixture of **19α**, **19γ** and **15** (1.4 mg, 4 mol%, 11:14:75). ^{1}H NMR (400 MHz, [D_6]DMSO): $\delta = 0.80$–0.83 (m, 18H), 1.05–1.71 (m, 52H), 1.84–1.99 (m, 1H), 2.19–2.33 (m, 3H), 3.17–3.49 (m, 53H), 3.66–3.70 (m, 1H), 3.80–3.82 (m, 1H), 4.25–4.26 (m, 1H), 4.47–4.48 (d, $J = 5.6$ Hz, 2H), 5.63 (s, 1H), 6.53–6.54 (d, $J = 8.8$ Hz, 2H), 6.94 (t, $J = 6.4$ Hz, 1H), 7.58–7.59 (d, $J = 8.4$ Hz, 2H), 9.24 ppm (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO): $\delta =$ 14.10, 19.58, 19.65, 19.72, 22.61, 22.71, 22.66, 24.34, 24.45, 24.76, 26.00, 27.95, 29.81, 29.85, 32.79, 29.34, 29.46, 29.52, 29.63, 29.68, 31.89, 37.26, 37.36, 37.39, 37.45, 37.50, 39.32, 26.25, 30.63, 38.70, 39.38, 46.25, 52.50, 65.63, 80.63, 111.88, 129.38, 166.96, 170.63 ppm; HRMS (ESI): m/z: calcd for $C_{82}H_{145}O_{19}N_9$ $[M-2H+3Na]^+$: 1627.0193; found: 1627.0189; $[M-H+2Na]^+$: 1605.0374; found: 1605.0337.

FA-PEG$_{570}$-tetraether 20: A solution of FA (**18**; 11 mg, 0.025 mmol, 2.5 equiv) in dry DMSO (3 mL) was heated until all material was dissolved. TBTU (16 mg, 0.05 mmol, 5 equiv), DIEA (8 μL, 0.05 mmol, 5 equiv) and a solution of **17** (17 mg, 0.01, 1 equiv) in dry DMSO (4 mL) were added at room temperature under a nitrogen atmosphere. After 24 h at 40°C in the dark, the reaction mixture was lyophilised. The residue was purified by dialysis (MWCO = 1000) against DMSO to give **20** as yellow solid mixture of **20α**, **20γ** and **17** (11.6 mg, 56 mol%, 47:44:9). ^{1}H NMR (400 MHz, [D_6]DMSO): $\delta = 0.74$–0.84 (m, 31H), 0.98–1.58 (m, 89H), 1.69–1.86 (m, 3H), 1.92–2.06 (m, 9H), 2.37–2.40 (m, 4H), 3.11–3.68 (m, 64H), 4.48–4.51 (m, 3H), 4.55–4.57 (m, 2H), 4.58–4.63 (m, 1H),

6.60–6.62 (m, 2H), 6.69–6.71 (m, 2H), 6.94 (m, 1H), 7.25 (m, 2H), 7.43–7.45 (m, 2H), 7.71–7.80 (m, 2H), 7.80 (m, 1H), 8.67 (s, 1H), 8.68 ppm (s, 1H);^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 17.23, 18.54, 19.62, 19.92, 22.00, 22.77, 24.54, 27.85, 29.46, 32.62, 37.31, 39.31, 47.85, 48.15, 59.69, 70.15, 115.42, 115.97, 136.32, 137.01, 144.51 ppm. LRMS (ESI): m/z: 2170 $[M]^-$.

Transfection assays: Adherent HeLa cells were seeded onto a 24-well plate in RPMI-FF (folate free; 500 μL) and with 10% foetal calf serum (FCS) at 100000 cells per well 24 h before transfection and incubated overnight in a humidified 5% CO_2 atmosphere at 37°C. For transfection, plasmid DNA (4 μg) encoding the luciferase reporter gene was mixed with the appropriate amounts of cationic liposomes depending on the charge ratio tested in a RPMI-FF medium without FCS. During the complexation time, cells were washed twice with PBS 1X and then preserved in the RPMI-FF medium (450 μL) not supplemented with FCS. The lipoplexes were added to the cell cultures 30 min later for competitive assays, various amounts of folate (1, 10, 25 and 50 nM) were also rapidly added to the medium. After 4 h, standard DMEM medium (2 mL) supplemented with FCS and folate was added to each well. Luciferase activity of the transfected cells was measured 48 h after transfection by using a chemiluminescent assay (Promega, Charbonnière, France). Measurements were carried out as described by the manufacturer. After rinsing twice in PBS 1X, cells were lysed with Lysis Buffer (200 μL, Promega) for 15 min. The supernatant was then distributed into the wells of a 96-well opaque plate. Luciferase activity in the supernatant was quantified with a luminometer (MLX Microtiter Plate Luminometer, Dynex, Gyancourt, France) to integrate light emission over a 15 s reaction period. In parallel, the total protein concentration was determined by using the BC Uptima protein assay (Interchim, Montluçon, France). The luciferase activity of each sample was normalised to relative light units (RLU) per mg of extracted protein. The total RLU mg^{-1} of protein obtained by summing up the RLU values of eight wells of microtitre plates, were plotted on the y axis. Each data point indicates the mean value of the total RLU mg^{-1} of protein from at least three experiments and the bars indicate the standard deviation of this mean.

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